

Nonmonotonic Behavior of the Dose Dependence of the Radiation Effect on Cells In Vitro Exposed to Pulsed Laser Radiation at $\lambda = 820$ nm

Tiina I. Karu, PhD,^{1*} Ludmila V. Pyatibrat, MS,¹ and Tatyana P. Ryabykh, PhD²

¹Laser Technology Research Center, Russian Academy of Sciences, Troitsk, Moscow Region, Russia

²N.N. Blokhin Cancer Research Center, Russian Academy of Medical Sciences, Moscow, Russia

Background and Objective: In recent years, clinical low-intensity laser therapy practice has used pulsed radiation, mainly from semiconductor lasers. Experimental works devoted to the study of relationships between biological and clinical effects and parameters of pulsed radiation are practically absent.

Study Design/Materials and Methods: The radiation source was a laser diode emitting at 820 nm (292 and 700 Hz, duty factor 80%; doses from 7 J/m² to 5 × 10⁵ J/m²; intensities 4, 12, 51, 152, 633, and 1,900 W/m²; irradiation time from 1 to 30 s). Four biological models were used: nucleated cells of murine spleen (splenocytes) and bone marrow (karyocytes), murine blood, and HeLa cells cultivated in vitro. The intensity of luminol-amplified chemiluminescence (in case of murine models) and the adhesion of HeLa cell membranes were measured as a function of the irradiation dose.

Results: Within the wide exposure dose range used we obtained seven maxima in the dose vs. biological effect curves: at fluences near 20, 1 × 10², 3 × 10², 8 × 10², 3 × 10³, 1 × 10⁴, and 3 × 10⁴ J/m². The peaks coincided for all four models.

Conclusion: The dose curves obtained with different cellular systems are of the same type and are characterized by seven peaks in the dose interval studied (7 J/m² to 5 × 10⁵ J/m²). *Lasers Surg. Med.* 21:485–492, 1997. © 1997 Wiley-Liss, Inc.

Key words: HeLa cells; low-power laser therapy; murine blood; murine bone marrow; murine splenocytes

INTRODUCTION

In recent years, clinical low-intensity laser therapy practice has used, in addition to continuous wave (CW) He-Ne laser radiation ($\lambda = 632.8$ nm), pulsed radiation, mainly from semiconductor lasers [1,2].

The dose dependencies of the stimulative effects of CW light on biochemical reactions have been studied using different radiation wavelengths and cellular systems. These dependencies are in principle of the same type and have a bell-like shape with a threshold, a sharp peak, and a

decay phase [3]. When studying the effect of CW radiation at $\lambda = 632.8$, 1,066, and 1,286 nm on the growth of *Escherichia coli*, it has been found that within a broad range of doses (from 8 J/m² to 2 × 10⁵ J/m²) there exist not one but two bell-shaped dose curves; the peak of one of them falls within a low-dose region (around 50 J/m²),

*Correspondence to: Prof. T. Karu, Laser Technology Research Center, 142092 Moscow Region, Troitsk, Russia. E-mail: Karu@lls.isan.troitsk.ru

Accepted 21 July 1997

whereas that of the other falls within a region of higher doses (10^4 J/m²) [4]. It has been assumed that at the root of the existence of these two stimulative dose regions are two different mechanisms governing the interaction between radiation and the primary photoacceptors, the cytochromes *bd* and *bo* [4,5].

Reports especially devoted to the study of the relationships between various biological and clinical effects and individual parameters of low-intensity *pulsed* radiation are very few in number (e.g., [6–12]). The present investigation is aimed at studying the dose dependencies of biological characteristics of cells exposed to pulsed GaAlAs laser radiation at $\lambda = 820$ nm ($f = 292$ and 700 Hz, pulse duration 2.74 and 1.14 ms, duty factor, i.e., pulse period-to-pulse duration ratio, 80%), widely used in clinical practice [1]. It has been shown as a result of this investigation that the dose curves obtained with different cellular systems are of the same type and are characterized by seven maxima in the dose interval studied (from 7 J/m² to 5×10^5 J/m²).

MATERIALS AND METHODS

The following four biological models were used in this work: nucleated cells of the spleen (splenocytes) and bone marrow (karyocytes) of 2-month-old male mice of the *A/Sn* strain, blood of 1.5- to 2-month-old male mice of the *A/Sn* strain, and a *HeLa* cell culture at the logarithmic growth stage. The mice were obtained from a local breeding colony "Stolbovaya" and kept in standard conditions.

Objects of Irradiation

For preparation of the suspension of splenocytes and keratinocytes, the mice were killed by cervical dislocation always at the same time (12 P.M.) to avoid circadian fluctuation in the activity of phagocytic cells.

A suspension of spleen cells or bone marrow cells was prepared by gentle homogenization of the organs in a glass Potter homogenizer at 0°C in 5 ml of Medium 199. The cells were centrifuged at 200*g* for 10 min, then resuspended in an ice bath in 0.83% NH₄Cl in 0.05M Tris buffer pH = 7.4 (10 ml solution per 10⁸ cells), and incubated in this solution for 5 min in an ice bath. The cells were again centrifuged at 200*g* for 10 min and resuspended in 4 ml of Medium 199 supplemented with 10% calf embryo serum (Institute of Poliomyelitis and Virus Encephalitis, Moscow). Counts of viable

nucleated cells were performed with a hemocytometer, and a suspension of 4×10^6 cells/ml was prepared for irradiation. In parallel with every irradiation experiment, smears for determining the cellular composition were prepared. The smears were fixed with methyl alcohol and stained with azureosin. Every series of experiments included a control experiment with *Candida albicans* (object of phagocytosis) as described in Kanu et al. [14]. These experiments served as a quality control of the cellular suspension subjected to irradiation.

Murine blood samples were taken from the submaxillary vein at 12 P.M. and diluted in a ratio of 1:10 with Medium 199 with 5% of embryonic calf serum (Institute of Poliomyelitis and Virus Encephalitis, Moscow) added. This solution was used for the irradiation.

HeLa cells, initially obtained from the Institute of Virology, Moscow, Russia, were grown in closed scintillation vials in 2 ml Medium 199 with the addition of 10% bovine serum (Institute of Poliomyelitis and Virus Encephalitis, Moscow) and 100 units/ml of penicillin and streptomycin. Cell viability was assessed by the trypan blue exclusion test, and all experiments were performed with cell suspensions demonstrating >85% viability by this criterion. Cells were grown in monolayer during 72 h and carefully harvested using 0.02% Versene solution; then a suspension was prepared in Medium 199 containing 10% of bovine serum (6.5×10^5 cells/ml). This suspension was used for experiments.

Light Source and Irradiation Procedure

The cellular suspension samples were irradiated with a Model Biotherapy 3ML GaAlAs laser (Omega, London, U.K.) 292 or 700 Hz in pulse repetition frequency (pulse duration, respectively, 2.74 and 1.14 ms, duty factor 80%), 820 nm in wavelength, and 15 or 50 mW in power output. If needed, the laser beam intensity was reduced with one or two neutral light filters. The power density was measured with a power meter Model 3 (Omega, London, U.K.). The beam intensity values used were 4, 12, 51, 152, 633, and 1,900 W/m² and exposure times ranged from 1 to 30 s, which made it possible to vary the radiation dose over a wide range (from 7 J/m² to 5×10^5 J/m²). The samples were placed in round glass sample cells (volume 130 μ l, inside diameter 0.7 cm, suspension layer thickness 0.4 cm) and irradiated in a dark room from above, so that the laser beam completely covered the sample surface. The

sample cells were made as follows. Two glass rings with ground edges were stuck on a microscope slide with a 5-cm distance between them. Both wells were filled with cell suspension. One of the wells was irradiated, and the other was used as control. The cell suspension exactly filled vials to the brim to avoid formation of a meniscus. Optimal irradiation condition (shape and dimensions of the vial, number of cells per vial) were developed in a special series of experiments.

Measurement of Chemiluminescence

Just after the irradiation, the samples were transferred to chemiluminometer tubes containing 100 μ l of luminol and 100 μ l of Medium 199. Luminol (Serva, Germany) was used as 1 mM solution in Na phosphate-buffered saline, pH 7.2. Kinetic curves of chemiluminescence were recorded during 40 min with a fully automated and computer-connected Model CL-3606 chemiluminometer (Dialog, Moscow, Russia). Kinetic curves of spontaneous chemiluminescence of nonirradiated cell suspensions (control experiment) were measured in the same way. Chemiluminescence measurements were used earlier to study continuous wave CW [14,10,11] and pulsed laser light [6,7,10–12] effects on murine splenocytes [7,10,12,14] and human blood [6,9,11,12].

Measurement of Cell–Glass Adhesion

Immediately after irradiation, the glass slide with samples of cell suspension was put into the thermostat (37°C) for 30 min. Then, the nutrient medium was removed, and the glass wells were washed with Hanks' solution to remove all nonattached cells. The attached cells were trypsinized, and their number was counted by a hemocytometer. The results presented are the mean \pm SEM of at least three experiments with five vials per point per experiment. In control samples, during a 30-min incubation, $43 \pm 2\%$ of cells attached to the glass. This value was used by data normalization. Cell–glass adhesion measurements were used earlier to characterize the dependence of cell membrane adhesive properties on CW light wavelength (action spectrum) and dose [13].

Statistics

Each data point on the figures is an average of five to ten independent experiments. The data were statistically processed by means of the *Statgraph* commercial statistical program package. A *P* value of $<.05$ was considered statistically significant.

RESULTS

Figure 1 presents some typical examples of luminol-amplified chemiluminescence (CL) of murine splenocytes (Fig. 1A), karyocytes (Fig. 1B), and diluted blood (Fig. 1C). Curve 1 in each part of Figure 1 characterizes the spontaneous CL (SCL) of intact cells. Irradiation can intensify the SCL (curve 2 in Fig. 1A–C). The kinetic curves of CL provide the basic information for further data processing. For evaluation of the effect of the irradiation, it appeared to be useful to process the data for a certain time point in the kinetic curves [6,9–12]. In the present work we used the same approach: Below the data in Figures 2 and 3 are presented for time point 25 min (Fig. 1). Also, the data below will be normalized, i.e., values of CL of irradiated samples to SCL of intact samples are presented. The normalized data allow comparison of the irradiation effects of different models.

The cells under study were characterized by a high ratio between phagocytosis-induced CL and SCL (Fig. 1D–F, curves 3 and 1, respectively). Note also that the CL of cells treated with *Candida albicans* (object of phagocytosis) was in all cases much higher than the CL of irradiated cells (compare curve 2 in Fig. 1A–C and curve 3 in Fig. 1D–F).

Irradiating the cellular suspension samples with pulsed radiation within a wide range of doses (fluences) allowed the following regularities to be revealed. The chemiluminescence of the splenocytes irradiated at $\lambda = 820$ nm and $f = 292$ Hz increased and decreased alternately as the fluence was raised (Fig. 2). Individual bell-shaped curves obtained with different radiation intensities and exposure times formed waves whose peaks coincided when the doses were the same (see, for example, peaks II, III, and IV in Fig. 2). Within the dose range used, we obtained seven peaks whose exact parameters are listed in Table 1.

A similar regularity was also observed to occur in the case of irradiation of blood samples at $f = 700$ Hz (Fig. 3A). As can be seen from this figure, there are also seven peaks within the given fluence range, the locations of which are the same as in Figure 2 (Table 1). As with Figure 1, the location of the peaks is independent of the radiation intensity and exposure time and is a function of the exposure dose alone (see, for example, peaks III, IV, V, and VI in Fig. 3A). In other words, the reciprocity rule holds here.

In two cases (CL of murine karyocytes and adhesion of HeLa cell membrane), the measure-

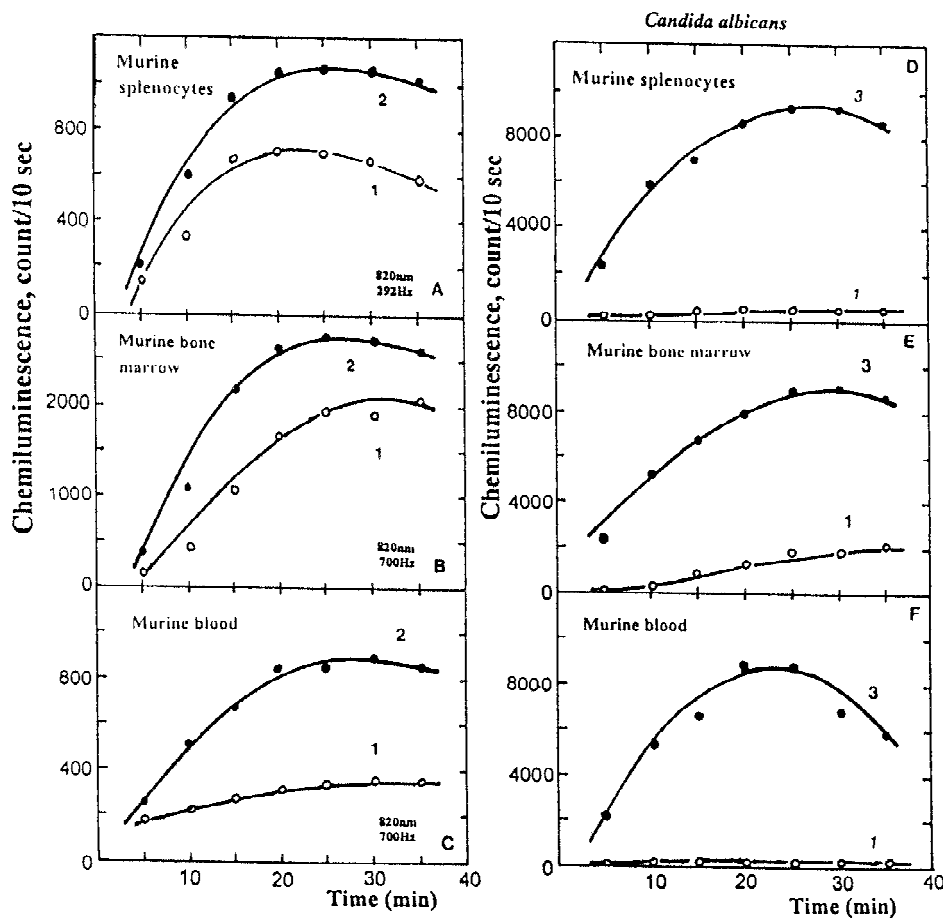


Fig. 1. Kinetic curves of chemiluminescence of mouse (A,D) splenocytes, (B,E) karyocytes, and (C,F) blood after (A–C) irradiation or (D–F) treatment with *Candida albicans*. Curve 1 denotes everywhere the spontaneous chemiluminescence of intact cells; curve 2, the CL after irradiation of samples in the dose 800 J/m^2 ($I = 51 \text{ W/m}^2$, $f = 292$ or 700 Hz); and curve 3, the CL induced by treatment with *C. albicans* (5×10^7 particles/ml). The measurement error is $\leq \pm 5\%$.

ments did not cover the entire range of fluences (Fig. 3B). It should also be noted that the radiation effects with these models were weaker (up to 140% relative to the control) as compared with the data presented in Figure 2 and 3A (up to 240%). Worthy of notice is the fact that peaks in the dose curves of Figure 3B almost coincide with the peaks of Figure 2 and 3A (see Table 1).

DISCUSSION

In the present work we measured the dose dependencies of the effects of 820-nm pulsed laser radiation on, first, the oxidative metabolism of mouse splenocytes, karyocytes, and blood, and second, the adhesive properties of the plasma membrane of HeLa cells.

Chemiluminescence measured in our experiments reflects the oxidative metabolism of phagocytosing cells (e.g., neutrophils, eosinophils,

monocytes, macrophages) and is associated with the activity of the membrane-bound enzyme, NADPH-oxidase [15–17]. NADPH-oxidase uses NADPH as an electron donor to reduce oxygen to superoxide anion, O_2^- [16]. CL is proportional to the amount of the reactive oxygen species produced by the phagocytosing cells and in particular, to the rate of which H_2O_2 is produced as a result of the dismutation of anion O_2^- [16]. NADPH-oxidase has been found in all phagocytosing cells and recently also in some nonphagocytic cells like B and T lymphocytes and fibroblasts [17]. The activity of NADPH-oxidase is low in intact cells but can increase rapidly in response to various stimuli. In other words, the chemiluminescence response kinetics reflect the process of NADPH-oxidase activation at its early stages [16,17].

In the experiments with HeLa cells, we measured the effect of 820-nm pulsed laser irradiation

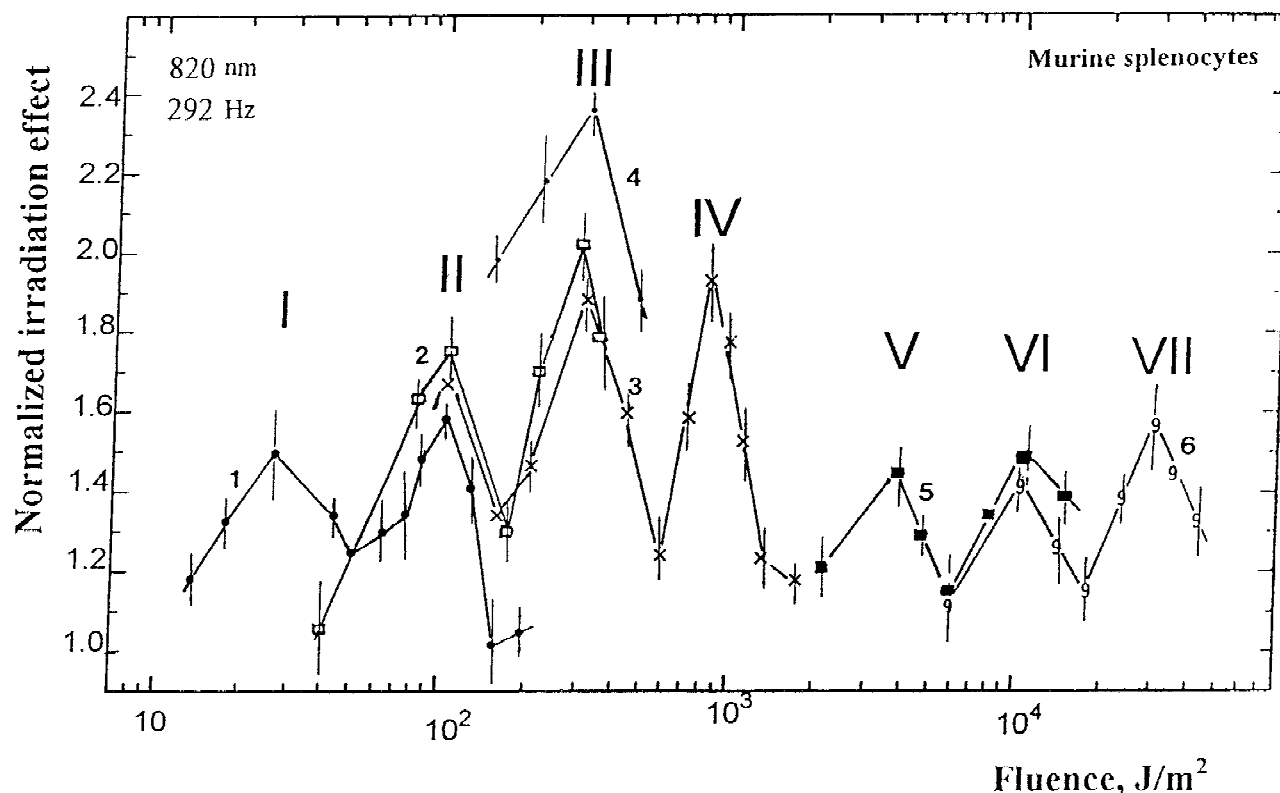


Fig. 2. Stimulation of chemiluminescence of murine splenocytes as a function of the fluence of laser radiation ($\lambda = 820$ nm, $f = 292$ Hz) differing in intensity: 1–4 W/m²; 2–12 W/m²; 3–51 W/m²; 4–152 W/m²; 5–633 W/m²; 6–1,900 W/m².

on the ability of the cells to attach themselves to the growth substrate. In other words, the action of radiation upon the formation of new contacts between the cell membranes and glass was studied. All the cell-matrix and cell-cell adhesion molecules isolated and characterized so far belong to glycoproteins, proteoglycans, and glycolipids [24], i.e., to the classes of molecules that do not absorb near-infrared (IR) radiation. One should note that the understanding of the molecular adhesion mechanisms is still fragmentary. Also, the mechanism by which visible and near-IR radiation acts on the plasma membrane of HeLa cells and changes its adhesive properties is still to be revealed. It was suggested on the basis of studies into the action spectra of HeLa membrane adhesion that this action could not be direct but was associated with the redox activation of the mitochondrial respiratory chain [13]. On the other hand, redox systems were found not only in mitochondria but also in the plasma membranes of many eukaryotic cells [18], HeLa cells included [19,20]. This cell membrane electron transport system was shown to act like a NADH-oxidase incorporating a flavoprotein, cyt b_5 , and Fe/S pro-

tein. This oxidase is believed to have a significant role in modulating cell attachment and replication [18].

As a result of this investigation we found that the dose curves obtained with different cellular systems are of the same type and are characterized by seven peaks in the dose interval studied (from 7 J/m² to 5×10^5 J/m²). The location of the peaks was found to be independent of the radiation intensity and exposure time and depended only on the fluence.

This finding was unexpected in that the results obtained by two different techniques (measurements of chemiluminescence and membrane adhesion) for four different models turned out to be similar, although both techniques evaluated the biological parameters associated with the cell membrane.

Three out of the four model systems used (diluted murine blood and suspension of splenic cells and bone marrow) were mixtures of various cells. One can assume at first glance that exactly this fact explains the occurrence of several peaks in the dose curves. This is not true for two reasons. First, the NADPH-oxidase in all phagocytosing

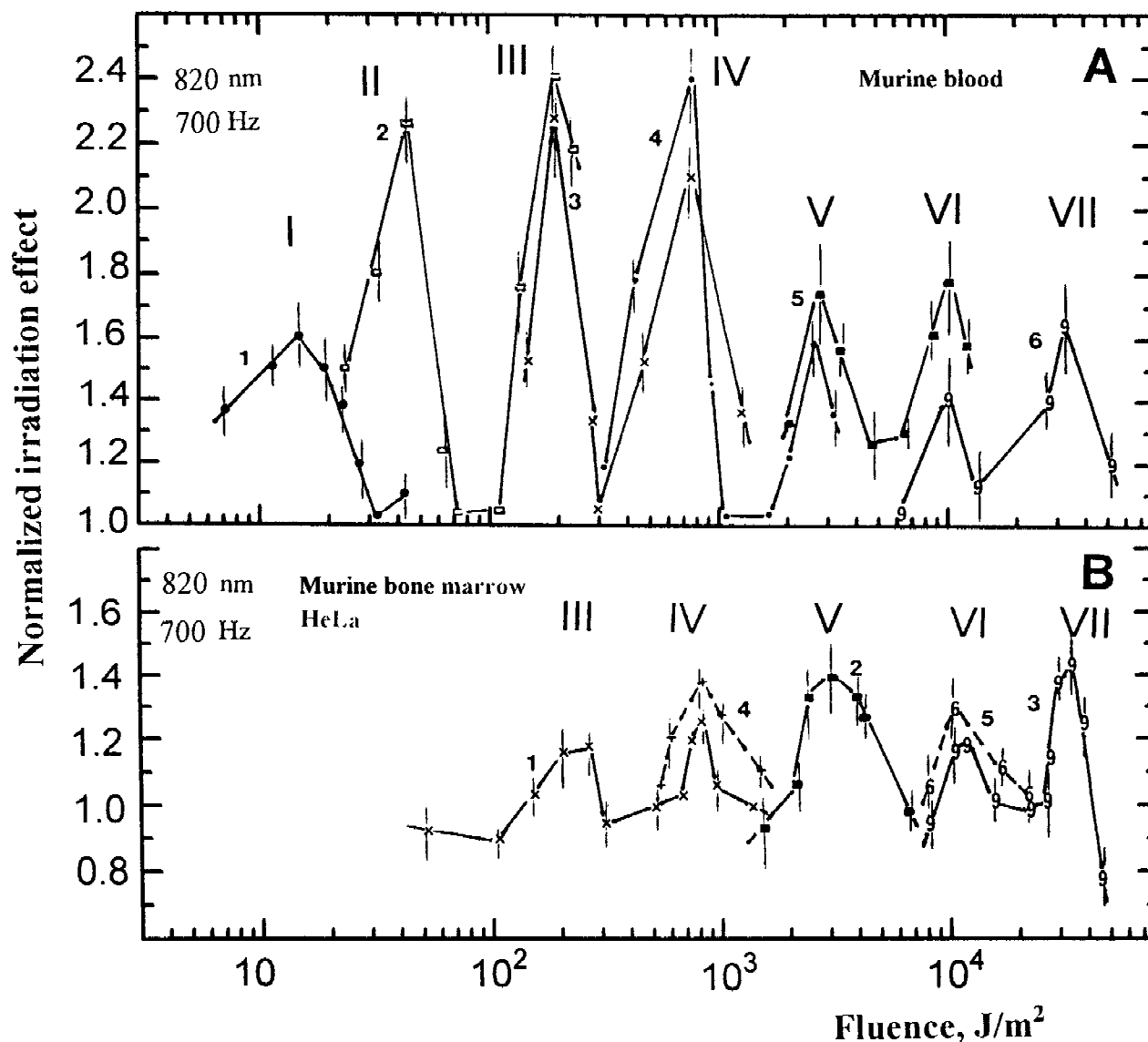


Fig. 3. Irradiation effect in exposure to laser radiation ($\lambda = 820$ nm, $f = 700$ Hz) differing in intensity. **A:** Stimulation of chemiluminescence of mouse blood (intensities: 1–4 W/m²; 2–12 W/m²; 3–51 W/m²; 4–152 W/m²; 5–633 W/m²; 6–1,900 W/m²). **B:** Enhancement of the adhesive properties of *HeLa* cell membranes (curves 1–3) or chemiluminescence of murine karyocytes (curves 4–5). Radiation intensities: 1 and 4–51 W/m²; 2–633 W/m²; 3 and 5–1,900 W/m².

cells is the same, both in structure and functioning principle [17]. Second, the *HeLa* suspension studied is not a mixture of different cells but is a homogeneous population.

It is reasonable to assume that the given nonmonotonic type of behavior of the cell responses as a function of the fluence is due to the pulsed character of the radiation used. The action of CW radiation is, as a rule, characterized by a single bell-shaped curve [3], or else by two such curves but associated with different mechanisms, presumably a photochemical and a thermal one [4].

As can be seen from Figure 2, as well as Table 1, the location of the peaks is independent of the radiation intensity and exposure time and is a function of the exposure dose alone. It means that one of the laws of photochemistry, namely, the reciprocity rule, holds here. This circumstance gives grounds to discuss a photobiological mechanism, which could be associated with the redox chains in cell membranes, NADPH-oxidase and NADH-oxidase. Pyridine and pyrimidine nucleotides, as well as flavoproteins, exhibit a reversible redox behavior in the regulation of various metabolic processes [21–23]. This reversible redox be-

TABLE 1. Laser Radiation Parameters Characterizing the Dose Curve Peaks

Peak λ, f	I			II			III			IV		
	I, W/m ²	t, s	D, J/m ²	I, W/m ²	t, s	D, J/m ²	I, W/m ²	t, s	D, J/m ²	I, W/m ²	t, s	D, J/m ²
820 nm	4	4	16	12	4	48	12	16	1.9×10^2	152	5	7.6×10^2
700 Hz							51	4	2×10^2	51	16	8×10^2
820 nm	4	6	24	12	9	1.1×10^2	152	2	3×10^2	51	16	8×10^2
292 Hz				51	2	1×10^2	12	25	3×10^2			
				4	25	1×10^2	51	6	3×10^2			
Peak λ, f	V			VI			VII					
	I, W/m ²	t, s	D, J/m ²	I, W/m ²	t, s	D, J/m ²	I, W/m ²	t, s	D, J/m ²	I, W/m ²	t, s	D, J/m ²
820 nm	633	4	2.5×10^3	633	16	1×10^4	1,900	16	3×10^4			
700 Hz	152	16	2.4×10^3	1,900	5	9.5×10^3						
820 nm	633	6	3.8×10^3	633	16	1×10^4	1,900	16	3×10^4			
292 Hz				1,900	6	1.1×10^4						

havior is due to the fact that the NAD and NADH pools, as well as the NADP and NADPH ones, are characterized by reciprocal changes in size. One can assume that irradiation causes transient changes in the NADP/NADPH or NAD/NADH pools. At the moment, this is a suggestion only, and further experimental work is needed to prove or disprove this assumption. Recall that recently a transient local heating mechanism explaining the inhibition of activity of NADPH-oxidase under pulsed 632.8-nm light was proposed [12].

The reason for the periodic nonmonotonic dose dependence in the present case and the mechanism at its root remain to be shown by further studies. At present we can conclude that under certain pulsed irradiation conditions at 820 nm (292 or 700 Hz, pulse duration 2.74 or 1.14 ms, duty factor 80%) the four models studied exhibited a nonmonotonic wave-shaped behavior of dose dependences. For all the models, seven similar maxima were found in the wide fluence interval under study (from 7 J/m² to 5×10^5 J/m²).

REFERENCES

- Baxter GD. "Therapeutic Lasers." Edinburgh: Churchill Livingstone, 1994.
- Karu T. "The Science of Low-Power Laser Therapy." London: Harwood Acad. Publ., 1997.
- Karu T. "Photobiology of Low-Power Laser Therapy." London: Harwood Acad. Publ., 1989.
- Karu T, Tiphlova O, Esenaliev R, Letokhov V. Two different mechanisms of low-intensity laser photobiological effects on *Escherichia coli*. J Photochem Photobiol B 1994; 24:155-161.
- Afanasyeva NI, Karu TI, Tiphlova OA. Oxidases bd and bo as primary photoacceptors at action of low-intensity visible (laser) radiation *Escherichia coli* cells. Dokl. Akad. Nauk (English transl., Doklady Biophysics) 1995; 345: 404-406.
- Karu T, Andreichuk T, Ryabykh T. Suppression of human blood chemiluminescence by diode laser radiation at wavelength 660, 820, 880 or 950 nm. Laser Ther 1993; 103-109.
- Karu T, Andreichuk T, Ryabykh T. Changes in oxidative metabolism of murine spleen following diode laser (660-950 nm) irradiation: effect of cellular composition and radiation parameters. Lasers Surg Med 1993; 13:453-462.
- El Sayed SO, Dyson M. Effect of laser pulse frequency on mast cell number and degranulation. Lasers Surg Med 1994; (Suppl 6):8-9 (abstr. 35).
- Karu T, Andreichuk T, Ryabykh T. On the action of semiconductor laser radiation ($\lambda = 820$ nm) on the chemiluminescence of blood of clinically healthy humans. Lasers Life Sci 1995; 6:277-282.
- Karu TI, Ryabykh TP, Antonov SN. Different sensitivity of cells from tumor-bearing organisms to continuous-wave and pulsed laser light ($\lambda = 632.8$ nm) evaluated by chemiluminescence test. I. Comparison of responses of murine splenocytes: intact mice and mice with transplanted leukemia EL-4. Lasers Life Sci 1996; 7:91-98.
- Karu TI, Ryabykh TP, Antonio SN. Different sensitivity of cells from tumor-bearing organisms to continuous-wave and pulsed laser radiation ($\lambda = 632.8$ nm) evaluated by chemiluminescence test. II. Comparison of responses of human blood: healthy persons and patients with colon cancer. Lasers Life Sci 1996; 7:99-105.
- Karu TI, Ryabykh TP, Letokhov VS. Different sensitivity of cells from tumor-bearing organisms to continuous-wave and pulsed laser radiation ($\lambda = 632.8$ nm) evaluated by chemiluminescence test. III. Effect of dark period between pulses. Lasers Life Sci 1997; 7:141-156.
- Karu TI, Pyatibrat LV, Kalendo GS, Esenaliev RO. Effects of monochromatic visible low-intensity light and laser irradiation on adhesion of HeLa cells in vitro. Lasers Surg Med 1996; 18:171-177.
- Karu TI, Ryabykh TP, Fedoseyeva GE, Puchkova NI. Induced by He-Ne laser radiation respiratory burst on phagocytic cells. Lasers Surg Med 1989; 9:585-588.
- Sbarra AJ, Strauss RR, eds. "Respiratory Burst and Its Physiological Significance." London: Plenum Press, 1988.
- Baggiolini M, Wyman MP. Turning on the respiratory burst. Trends Biochem Sci 1990; 15:69-72.
- Segal AW, Abo A. The biochemical basis of the NADPH-

- oxidase of phagocytes. *Trends Biochem Sci* 1993; 18:43–47.
18. Crane L, Morrè DJ, eds. "Plasma Membrane Oxidoreductases in Control of Animal and Plant Growth." London: Plenum Press, 1988.
 19. Sun IL, Crane FL, Grebing C, Löw H. Properties of a transplasma membrane electron transport system in HeLa cells. *J Bioenerg Biomembr* 1984; 16:583–595.
 20. Navas P, Sun IL, Morrè DJ, Crane FL. Decrease of NADH in HeLa cells in the presence of transferrin or ferricyanide. *Biochem Biophys Res Commun* 1986; 135: 110–115.
 21. Czochralska B, Bojarska E, Pawlicki K, Shugar D. Photochemical and enzymatic redox transformations of reduced forms of coenzyme NADP⁺. *Photochem Photobiol* 1990; 51:401–410.
 22. Ghisla S, Massey V. Mechanisms of flavoprotein-catalyzed reactions. *Eur J Biochem* 1989; 181:1–17.
 23. Gutman M, Bonomi F, Pagani S, Cerletti P, Kroneck P. Modulation of the flavin redox potential as mode of regulation of succinate dehydrogenase activity. *Biochim Biophys Acta* 1980; 591:400–408.
 24. Anderson H. Adhesion molecules and animal development. *Experientia* 1990; 46:2–13.